ORIGINAL ARTICLE

Influenza virus A(H1N1)pdm09 hemagglutinin polymorphism and associated disease in southern Germany during the 2010/11 influenza season

Valeria Falcone · Sibylle Bierbaum ·
Winfried Kern · Udo Kontny · Hartmut Bertz ·
Daniela Huzly · Marcus Panning

Abstract A novel influenza A virus emerged in early 2009 to cause the first influenza pandemic of the 21st century. Understanding the evolution of influenza virus is crucial to determine pathogenesis, vaccine efficacy, and resistance to antiviral drugs. In this study, we investigated the molecular evolution of influenza virus A(H1N1)pdm09 in the 2010/11 influenza season in southern Germany by sequence analysis of the influenza virus hemagglutinin gene from 25 patients with mild, moderate, and severe disease. Phylogenetic analysis revealed co-circulation of different genetic groups. The D222G mutation, which had previously been observed in severe cases, was not detected. Immunocompromised patients were not affected more severely than non-immunocompromised patients ($p > 0.05$), although longer shedding was observed in some of them. Interestingly, additional mutations and potential glycosylation sites were detected in samples from the lower respiratory tract in two patients, but not in the corresponding upper respiratory tract specimens. The H275Y mutation in the influenza virus neuraminidase gene, known to confer resistance to the neuraminidase inhibitor oseltamivir, was detected in one patient.

Introduction

The first influenza pandemic of the 21st century emerged in Mexico in March 2009 and was caused by a novel influenza A(H1N1)09 virus (A(H1N1)pdm09). In general, the course of the pandemic was moderate from a public-health perspective. However, a particular proportion of the human population including immunocompromised patients, children and pregnant women was at risk to develop severe disease.

Hemagglutinin (HA) and neuraminidase (NA) represent two major surface glycoproteins of influenza virus. The HA determines host-receptor tropism and constitutes the key immunogenic site for the human immune response. Since the beginning of the 2009 pandemic, A(H1N1)pdm09 rapidly evolved, and seven different clades/groups characterized by distinct molecular markers in the HA gene have been reported [23]. The D222G mutation in the receptor-binding domain of the HA gene has been linked to severe cases [10]. Of note, another mutation involving the same residue (D222E) was also observed but could not be associated with more severe cases [1]. Understanding the molecular evolution of influenza virus is therefore crucial to identify mutations that might be associated with a more virulent phenotype. The second surface glycoprotein, NA, facilitates the release of newly synthesized virions from infected cells [5]. Neuraminidase can be blocked by antiviral drugs such as oseltamivir and zanamivir, which act by interfering with the release of progeny virus, thereby preventing new rounds of infection. A single point mutation at

V. Falcone · S. Bierbaum · D. Huzly · M. Panning (✉)
Department of Virology, Institute for Medical Microbiology
and Hygiene, University Medical Center,
Hermann-Herder Str. 11, 79104 Freiburg, Germany
e-mail: marcus.panning@uniklinik-freiburg.de

W. Kern
Department of Medicine, University Medical Center,
Freiburg, Germany

U. Kontny
Center for Paediatrics and Adolescent Medicine,
University Medical Center, Freiburg, Germany

H. Bertz
Department of Haematology/Oncology,
University Medical Center, Freiburg, Germany

position 275 in the neuraminidase glycoprotein, resulting in a histidine-to-tyrosine shift, can confer resistance to oseltamivir [13].

Aim of this study was to investigate the molecular evolution of A(H1N1)pdm09 in the 2010/11 influenza season in southern Germany by sequence analysis of the HA gene of mild, moderate, and severe influenza cases. Particular attention was paid to immunocompromised patients. Moreover, the occurrence of resistance to oseltamivir, the only drug currently recommended for prophylaxis in high-risk groups, was determined.

Materials and methods

Respiratory samples

Respiratory specimens were obtained from paediatric and adult patients hospitalized at the Freiburg University Medical Centre as well as from outpatients. All had influenza-like illness, including fever, cough, and/or sore throat, as judged by the treating physician. Respiratory specimens comprising nasopharyngeal aspirates (NPA), tonsillo-pharyngeal flocked swabs collected in 0.5 ml viral transport medium (Copan, Brescia, Italy), or bronchoalveolar lavage fluids (BAL) were analysed at the Department of Virology. Testing of patient samples was approved by the institutional review board of Freiburg University.

Molecular analysis

In brief, nucleic acids were extracted using a QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany) on a QIACube robot (QIAGEN) according to the manufacturer's instructions. In 2010/2011, a broadly reactive multiplex PCR approach was chosen to detect influenza viruses A and B as well as other relevant respiratory viruses. Samples were analysed using FTD Respiratory Pathogens version 08/2010 (Fast-track Diagnostics, Junglinster, Luxemburg) as recommended. The assay utilizes 5'nuclease technology (TaqMan) and employs a 5-tube multiplex one-step real-time RT-PCR approach. In tube 1, real-time RT-PCR for influenza virus A, A(H1N1)pdm09, influenza virus B, and rhinovirus are combined. Tube 2 contains reagents for parainfluenza viruses 2, 3, and 4, and brome mosaic virus (BMV) as a PCR inhibition control; tube 3, for coronavirus 229E, coronavirus NL63, coronavirus OC43, and coronavirus HKU1; tube 4, for respiratory syncytial virus A/B, adenovirus, parechovirus, and enterovirus; and tube 5, for parainfluenza virus 1, human metapneumovirus A/B, human bocavirus, and *Mycoplasma pneumoniae*. The BMV inhibition control was added to each patient sample before nucleic acid extraction. An AgPath-ID One-Step RT-PCR-Kit (Invitrogen, Karlsruhe, Germany) was used for RT-PCR on an ABI 7500 real-time machine (Applied Biosystems, Wiesbaden, Germany). Cycling conditions were as follows: 50 °C for 15 min, 95 °C for 10 min followed by 40 amplification cycles of denaturation at 95 °C for 8 s and combined annealing/extension at 60 °C for 34 s.

For the detection of the oseltamivir-resistance-associated mutation H275Y, a commercially available assay (TIB-Molbiol, Berlin, Germany) was used according to the manufacturer's instructions.

Sequencing and phylogenetic analysis

The HA gene was amplified directly from clinical samples using the SuperScript ^{TM}III One-Step RT-PCR system (Invitrogen, Karlsruhe, Germany). In brief, a reaction volume of 50 μl contained 1x reaction buffer, 2 mM MgSO$_4$, 0.5 μM each primer, 2 μl enzyme mix, and 5 μl of purified nucleic acids. The primer sequences were as follows: H1N1_HA_F1, CCG CAA ATG CAG ACA CAT TA; H1N1_HA_R1, CCC ATT AGA GCA CAT CCA GAA [12]. Cycling conditions in a Veriti 96-well thermal cycler (Applied Biosystems, Weiterstadt, Germany) were 50 °C for 30 min and 94 °C for 2 min, followed by 33 amplification cycles at 95 °C for 15 s, 51 °C for 30 s, and 68 °C for 30 s. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) as recommended. Purified PCR products were sequenced directly using primers H1N1_HA_F1 (CCGCAAATGCAGACACATTA) and H1N1_HA_FSeq_902 (CAGACACCCAAGGGTGCTAT).

Sequences were aligned using BioEdit (Mega 4.1). Nucleotide sequence alignments were done using a ClustalW method with MEGA 4.1. Phylogenetic trees were constructed using the maximum-parsimony method. NCBI GenBank accession numbers for the sequences determined here are JX413801 to JX413833. Amino acids were numbered starting after the DTLC signal peptide. Potential N-linked glycosylation sites were predicted using the free software NetNGlyc 1.0.

Statistics

Data were analyzed using SPSS software version 19 (SPSS, Chicago, USA). Data were compared by Fisher's exact test, and p-values were deemed significant at the 0.05 level.

Results

Clinical and virological features

In order to detect molecular changes in the HA gene of influenza virus, 33 A(H1N1)pdm09 HA sequences

representing 25 individual patients were analysed. As shown in Table 1, 14 of the 25 patients (56 %) were immunocompromised or immunosuppressed, suffering from different underlying diseases. Virus isolates on MDCK SIAT-1 cells were obtained from 28 of the 33 respiratory samples (data not shown) [16]. The median age of the patients was 40 years (95 % confidence interval [CI], 22.9-44.3 years]. Based on clinical criteria, 11/25 (44 %) were regarded as mild (median age, 6 years; range, 1-55 years), 10 (40 %) as moderate (median age, 53 years; range 0.1-76 years) and 4 (16 %) as severe cases (median age, 47 years; range, 0.2-55 years) according to Zarychanski et al. [27]. All severe cases were hospitalized patients known to be at risk for severe disease (3 of these 4 patients were immunocompromised, and one of them was obese). Only 1 of the 11 immunocompetent patients and 3 of the 14 immunocompromised patients developed severe disease (Fisher's exact test, p=0.60).

In 7 (21 %) of the 33 samples (corresponding to 5 [20 %] of the 25 patients), co-infection with another respiratory virus was detected. Coronavirus OC43 and/or NL63 were detected in 3 of these 5 patients, RSV and human bocavirus were co-detected in the NPA of one, and RSV alone was detected in a pharyngeal swab from another patient. Clinically, the patient with RSV and bocavirus co-infection presented with mild disease; the remaining patients displayed moderate (n=3) to severe (n=1) symptoms. None of the patients showed co-infection with *Mycoplasma pneumoniae* as assessed by multiplex PCR.

Table 1 Baseline demographic, virological and clinical data of the study population

Patient characteristic	All patients (n= 25)	Disease classification*		
		Mild (n=11)	Moderate (n=10)	Severe (n=4)
Male	17	8	6	3
Female	8	3	4	1
Immunocompetent	11	6	4	1
Immunosuppressed	14	5	6	3
HSCT recipient	5	1	3	1
Chemotherapy	3	2	1	0
Solid organ transplant recipient	1	0	1	0
HIV infection	1	1	0	0
Genetic disorder	1	0	0	1
Other	3	1	1	1
Viral co-infection	5	1	3	1
Influenza virus shedding, >2 weeks	6	1	2	3
NA resistance mutation H275Y	1	0	0	1

*According to Zarychanski et al. [26]

Prolonged shedding (i. e., ≥ 2 weeks) of influenza virus in the respiratory tract was observed in 6 of the 25 patients (24 %; 3 with severe, 2 with moderate, and 1 with mild disease). A total of 5 out of 6 patients were immunocompromised, and 5 of the 25 patients (4 of them immunocompromised) received oseltamivir therapy.

Phylogenetic analysis

The HA sequences were closely related to each other and to the reference strain A/California/07/2009 (A/Cal/H1N1/09). The differences between German sequences and A/Cal/H1N1/09 ranged from 5 to 10 amino acids (Table 2). All of the viruses analysed displayed the amino acid changes P83S and S203T in the HA1 region as well as I321V and E 374 K in the HA2 region.

Phylogenetic analysis showed simultaneous co-circulation of influenza virus of groups 4, 5, 6, and 7 in southern Germany (Figure 1). Genetic groups were named according to the ECDC technical document of August/September 2011. In detail, 16 of the 33 sequences (48 %) belonged to genetic group 6, characterized by the double mutation D97N and S185T. Moreover, 3 of these 6 showed an additional S84I mutation (as influenza A/Delaware/AF21764/2010), and one of them had a P159S mutation. Of note, this particular strain was isolated from an influenza-vaccinated individual. Ten of the 33 sequences (30 %) belonged to genetic group 5, characterized by the mutations D97N, R205K, I216V and V249L. In this group, three also had the H138Q mutation. As observed by Piralla et al. [18], two sub-clusters were seen within this group. Group 4, characterized by amino acid mutations N125D included four sequences, all obtained from the same patient. Finally, 3 of the 33 sequences (9 %) clustered within group 7 and were characterized by the mutations S185T, S143G, and A197T. Double mutations characteristic of group 3 (A134T, S183P), and group 2 (N31D, S162N) were not observed. Severe, moderate, and mild cases were scattered throughout the phylogenetic tree.

Some of the observed amino acid substitutions involved the major antigenic sites of the HA molecule (Table 2).

Five potential N-glycosylation sites, typical of A/Cal/H1N1/09, corresponding to residues 23, 87, 276, 287, and 481, were also detected in the German sequences. However, in two patients, two additional glycosylation sites were detected at residue 119 (lysine to asparagine) in one case and at residue162 (serine to asparagine) in the other case. As shown in Table 2, JX413805 and JX413803 correspond to sequences obtained from the upper and lower respiratory tract of the same patient. Interestingly, the K119N mutation, representing an additional potential N-glycosylation site, was present only in the BAL sample. Moreover, the cycle threshold (Ct) value of the H1N1

Table 2 Observed HA amino acid mutations of mild, moderate, and severe cases in comparison to the vaccine strain A/Cal/H1N1/09. NCBI GenBank accession numbers and specimen type are shown. Highlighted sequences in the same colour are follow-up samples from the same patient

Severe ILI cases (JX41 3818–3830) — Moderate ILI cases (JX41 3808–3811)

Specimen types — Severe: 3818 BAL, 3802 NPS, 3821 NPS, 3822 BAL, 3805 NPS, 3803 BAL, 3804 NPS, 3808 NPS, 3830 BAL; Moderate: 3808 NPS, 3812 NPS, 3828 BAL, 3814 BAL, 3825 NPS, 3817 NPS, 3801 NPS, 3833 NPS, 3810 NPS, 3831 NPS, 3829 NPS, 3806 NPS, 3811 NPS

Position	Antigenic sites	A/Cal 07/09	3818	3802	3821	3822	3805	3803	3804	3808	3830	3808	3812	3828	3814	3825	3817	3801	3833	3810	3831	3829	3806	3811
56		N					S	S																
74	Cb	S																						
77		S	F	F		F																		
83		P	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
84		S																	I	I				
97		D					N	N	N	N	N	N	N	N	N				N	N	N	N	N	N
119		K						N																
125	Sa	N	D	D	D	D																		
128		S																						
130		K				R																		
138	Ca2	H												Q	Q	Q	Q							
143		S													G	G	G							
159	Sa	P																						
162	Sa	S																						
178		G																						
180		H																	Q	Q				
183	Ca1	S																						
185	Sb	S					T	T			T	T	T	T			T	T	T	T			T	T
186	Sb	A																						
190	Sb	S																						
197		A													T		T	T						
203	Ca1	S	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
205	Ca2	R							K	K				K			K				K	K		
216	Ca2	I					K		V	V							V	V			V	V		
221		R							K	K														
249		V							L	L					L		L				L	L		
300		I											T											
310		T																	S	S				
321		I	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
344		M				L																		
356		E														A								
374		E	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K
451		S					N	N			N	N	N	N			N	N	N	N			N	N
509		R	K	K	K	K																		

Mild ILI cases

All specimens: NPS

| Position	Antigenic sites	A/Cal 07/09	3809	3827	3813	3826	3824	3823	3820	3815	3819	3832	3816
56		N											
74	Cb	S	N										
77		S											
83		P	S	S	S	S	S	S	S	S	S	S	S
84		S				I							
97		D	N	N	N	N	N	N	N	N	N	N	N
119		K											
125	Sa	N											
128		S						P					
130		K											
138	Ca2	H					Q		Q				
143		S											
159	Sa	P											S
162	Sa	S									N		
178		G											
180		H											
183	Ca1	S			P								
185	Sb	S		T		T		T		T	T	T	T
186	Sb	A					T						
190	Sb	S				G							
197		A											
203	Ca1	S	T	T	T	T	T	T	T	T	T	T	T
205	Ca2	R	K		K		K		K				
216	Ca2	I	V		V		V		V				
221		R											
249		V	L		L		L		L				
300		I											
310		T											
321		I	V	V	V	V	V	V	V	V	V	V	V
344		M											
356		E					A		A				
374		E	K	K	K	K	K	K	K	K	K	K	K
451		S		N	N			N		N	N	N	N
509		R											

real-time RT-PCR for the throat swab was 26, compared to 19 for the BAL sample, suggesting a higher concentration of the virus in the lower than in the upper respiratory tract.

Similarly, in another severe case, the Ct value for the BAL sample obtained 20 days after onset of disease was lower (Ct 21) than that for the nasopharyngeal swab (Ct 29). Also in this case, two additional amino acid changes (K130R and M344L, GenBank accession number JX483122) were detected in the BAL sample.

Treatment with oseltamivir was reported in 5 of the 25 patients (4 immunocompromised patients and 1 obese patient). The oseltamivir-resistance-associated mutation H275Y was detected in 1 (20 %) of the 5 treated patients, a one-year-old child with a genetic disorder, who was treated for 21 days with oseltamivir. He recovered fully from

Fig. 1 Phylogenetic analysis of HA genes of circulating influenza viruses in southern Germany during the 2010/11 influenza season. Reference influenza virus strains are shown in bold. Characteristic mutations of the different genetic groups are indicated by red boxes. Sequences of our study are indicated by NCBI GenBank accession number
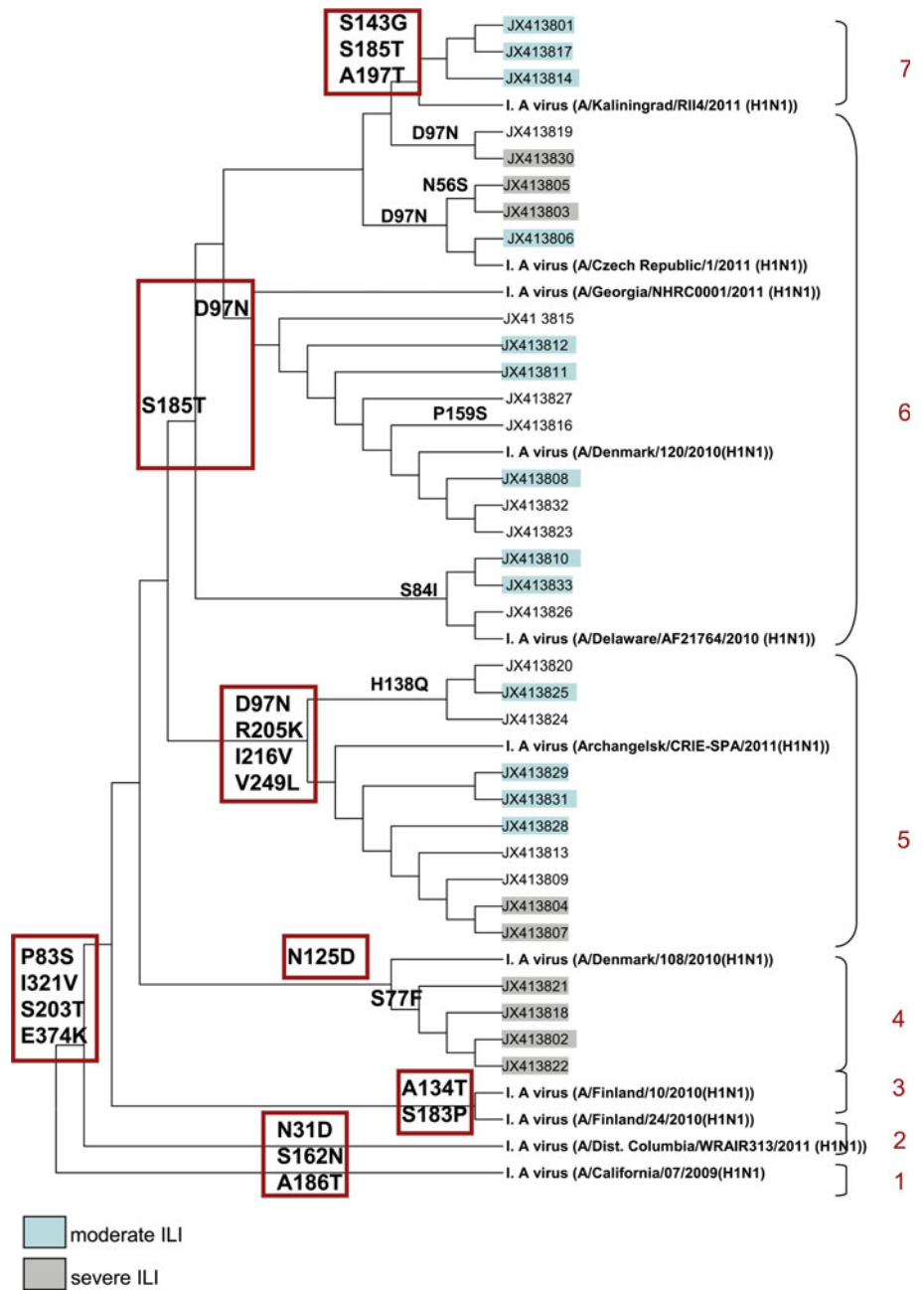
influenza despite development of oseltamivir resistance and prolonged shedding of resistant virus for > 2 weeks.

Discussion

In this molecular study, we were able to show that influenza viruses circulating in southern Germany in the first post-pandemic season differed relatively little from each other and from the vaccine strain A/Cal/H1N1/09.

Some of the amino acid substitutions that were observed involved the major antigenic sites of the HA molecule. Five classical antigenic sites (Sa, Sb, Ca1, Ca2, and Cb)

have been described in the HA of seasonal influenza H1N1 virus [3, 24], all located in the globular head of HA. Recent studies have also described some important antigenic sites in the stem region of HA [25].

All viruses analysed here had the amino acid changes P83S and S203T in the HA1 region and I321V and E374K in the HA2 region. As reported by others [8, 18, 19], and as published in the ECDC report released in August/September 2011 [23], co-circulation of different genetic groups was observed. Group 6, characterized by mutations D97N and S185T, was the dominant H1N1 lineage (48 %), followed by group 5 (33 %; D97N, R205K, I216V), group 4 (12 %; N125D) and group 7 (9 %; S185T, S143G, A197T).

Although a number of mutations have been reported in circulating A(H1N1)pdm09, they have not significantly affected virus antigenicity and pathogenicity as demonstrated by *in vitro* studies [26]. Clinically, the mutations D222G and N have been associated with a more virulent phenotype [10]. However, recent studies show that within the current A(H1N1)pdm09 HA framework, the effect of the 222 mutation on receptor binding appears to be less dramatic when compared to the 1918 influenza A(H1N1) virus HA framework, since the binding preference for α2-6 sialylglycans is still maintained [26]. Also, other mutations, such as the double mutation N125D and E374K, have been associated with a more virulent phenotype. This double mutation has been associated with several breakthrough infections despite influenza vaccinations and was identified in some fatal cases [2]. Moreover, it was associated with decreased antibody recognition in vaccinated individuals [21]. The German HA sequences carrying this double mutation originated from an unvaccinated immunocompromised individual with severe illness. Due to the lack of serum specimens, we could not analyze the ability of antibodies to recognize the hemagglutinin of A(H1N1)pdm09 in our study. However, immune escape from the vaccine strains might be an issue of concern.

No D222G change was observed here. In our study, no association of a specific amino acid change with severe illness could be observed. However, in two patients, differences between the HA sequence could be detected in viruses isolated either from the upper respiratory tract (URT) or the lower respiratory tract (LRT). In particular, two additional mutations (K130R-M344L in one case and K119N-I216K in the other case) were identified only in the LRT. Moreover, analysis of potential glycosylation sites revealed that the K119N mutation provides an additional potential glycosylation site. Human influenza viruses carrying the K119N mutation show improved growth in eggs and appear to exhibit enhanced virulence in the mouse model [9, 14]. Glycosylation at position 119 is essential for improved virus protein yield in eggs [14]. Egg adaptation of human influenza viruses is known to increase their affinity for the 2,3-sialic acid (SA) receptor and concomitantly impairs their ability to bind to 2,6-SA [11]. Although it is clear that influenza virus tropism depends on several viral and host factors and not only on HA specificity, the presence of the K119N mutation exclusively in the virus isolated from the LRT might indicate a more efficient binding/replication of viruses carrying this mutation to/in cells expressing 2,3-SA. Importantly, 2,3-SA is known to be found in abundance in the lower respiratory tract [20].

Of note, the Ct value of the A(H1N1)pdm09 real-time RT-PCR from the nasopharyngeal swab was higher than that of the BAL sample carrying the mutation (Ct 26 versus Ct 19). Since BAL samples are usually significantly more diluted than nasopharyngeal swabs, the observed difference suggests a real replication advantage of the strain harboured in the LRT. Virus histochemistry studies could be used to analyze the pattern of binding of mutants to human respiratory tissue, as already suggested [6, 22]. Moreover, analysis of growth curves of the two isolates in different cell lines may reveal a possible replication advantage, at least in cell culture. Many patients with influenza have more than one viral agent, with reported co-infection frequencies as high as 20 % [4]. We detected co-infection with another respiratory virus in 7 out of 33 samples (21 %), corresponding to 5 of the 25 patients (20 %). The most frequent co-infecting agents were coronaviruses (5 of 7 samples), followed by RSV (2 of 7 samples) and human bocavirus (1/7). Disease resulting from co-infection with influenza virus and coronaviruses has been reported to be more severe [4], although the number of influenza virus and coronavirus co-infections is low [4, 15, 17]. Here, patients co-infected with coronaviruses showed moderate to severe disease, whereas co-infection with RSV and human bocavirus in one child resulted in mild disease.

Finally, the oseltamivir-resistance-associated mutation H275Y was detected only once in our study population. Although oseltamivir has been widely used in the 2009 pandemic and thereafter, cases of resistance have remained scarce to date. However, prolonged shedding of high levels of resistant influenza virus in individual cases poses the threat of spread into populations that are at risk and finally into the general population as resistant viruses retain fitness [7].

In concordance with other studies, our results underline the importance of monitoring influenza virus evolution and development of resistant viruses. Of note, the LRT might harbour more virulent variants than the URT. Immune escape of influenza virus in subsequent influenza seasons is an issue of concern, making continuous surveillance essential.

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